#19 APP

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Applicant:	José Halperin
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Examiner:	A. Decloux
Art Unit:	1644

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MaryDilys S. Anderson

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF DR. JOSÉ HALPERIN

1. I am an attending physician in the Department of Medicine at the Brigham and Women's Hospital and am an Associate Professor of Medicine at the Harvard Medical School. I have been involved with research on cell proliferation, its regulation and mitogenic signals triggered by activation of the complement system in general and in diabetes in particular for 15 years at the Laboratory for Membrane Transport of the Harvard Medical School.

2. I made the scientific observation that activation of the complement system induces cell proliferation by releasing growth factors and cytokines from cells such as endothelial cells. These cellular factors are released from the complement targeted cells via the complement pore known as membrane attack complex or MAC. These original observations have been published in several leading peer-reviewed articles.

3. This Declaration details seven experiments, all of which were carried out under my direct supervision and control.

4. I provide herein descriptions and results of experiments done using the methods of the invention to detect levels of glycated and nonglycated CD59 in urine, blood, and tissue samples. This Declaration provides evidence that the methods of the invention are enabled for tissues and fluids other than urine such as blood, in addition to being enabled for urine samples.

5. Each experiment described herein involved the detection of glycated CD59, the complement regulatory protein that inhibits formation of the MAC. The initial step in the experiments included the generation of an antibody that recognizes the glycated form of human CD59 but does not recognize the non-glycated form or other glycated proteins. To raise this antibody a peptide was synthesized encompassing the glycation site formed by amino acid residues lysine 41 and histidine 44 and containing a glycated lysine (K_{glu}) in position 41. In addition, two cysteine residues in the peptide were replaced by alanine residues to avoid formation of disulfide bridges (Figure 1). The peptide (termed $CD59_{36-49}$-$K41_{(glu)}$) was synthesized by solid phase methodology, purified by affinity chromatography, and the structure of the purified synthetic peptide confirmed by mass spectrometry.

6. Experiment 1 An anti-glycated human CD59 antibody was prepared. For this process, two rabbits were immunized with the human $CD59_{36-49}$-$K41_{(glu)}$ peptide and the antibody titer detected by ELISA using the same peptide as standard. Non-immune serum obtained before immunization was kept for negative controls. The rabbit serum demonstrating high levels of anti- $CD59_{36-49}$-$K41_{(glu)}$ was collected and the anti- $CD59_{36-49}$-$K41_{(glu)}$ specific immunoglobulin IgG fraction was purified by affinity chromatography using $CD59_{36-49}$-$K41_{(glu)}$ attached to a solid phase support

7. Experiment 2 The specificity of the anti-glycated human CD59 antibody was documented. Human CD59 was purified from human red blood cells and then glycated *in vitro* by exposure to 0.5M glucose for variable times. The specificity of the antibody was then documented by both Western blot analysis (Fig. 2A) and ELISA (Fig. 2B). Figure 2 shows that the anti-glycated CD59 antibody recognizes purified human CD59 after but not before glycation and does not recognize another glycated protein such as glycated albumin (purchased from Sigma Co and routinely used as a standard for

glycated proteins). Glycation in CD59 occurs in lysine 41 because the anti-glycated CD59 antibody did not recognized the human CD59 mutant (in which lysine 41 was replaced by alanine) after exposure to glucose for a similar time interval (not shown).

8. Experiment 3 The anti-glycated CD59 antibody was used to measure by ELISA the presence of glycated CD59 in human urine. An ELISA using an antibody against total CD59 was also applied to the samples and the results expressed as the ratio of glycated-CD59/Total CD59 (i.e. the relative amount of glycated CD59 in each urine sample). Urine samples were from non-diabetic and diabetic subjects. Figure 3 shows that glycated CD59 can be found in human urine and that it correlated well with the levels of glycated hemoglobin (HbA1C) in blood, the current clinical standard for assessment of glycemic exposure in diabetic patients.

9. Experiment 4 The anti-glycated CD59 antibody was used to measure by ELISA the presence of glycated CD59 in human plasma. An ELISA using an antibody against total CD59 was also applied to the samples and the results expressed as the ratio of glycated-CD59/Total CD59 (i.e. the relative amount of glycated CD59 in each plasma sample). Plasma samples from non-diabetic and diabetic subjects were obtained by centrifugation (for 5 minutes at 1000g) of a sample of blood treated with EDTA to avoid clotting. Figure 4 shows that glycated CD59 can be found in human plasma and that it correlated well with the levels of glycated hemoglobin (HbA1C) in blood, the current clinical standard for assessment of glycemic exposure in diabetic patients.

10. Experiment 5 The anti-glycated CD59 antibody was used to detect the presence of glycated CD59 in diabetic kidneys. The kidney samples were paraffin-embedded renal biopsies from diabetic patients that underwent a renal biopsy because of renal failure and to detect diabetic nephropathy (also known as glomerulosclerosis). The samples were obtained from a collection of renal biopsies from the Pathology Department of Brigham and Women's Hospital. The paraffin blocks were sectioned, paraffin removed by standard methods, and the thin sections were stained with anti-glycated CD59 antibody, with anti-MAC antibody, and with the IgG fraction of the rabbit serum extracted before immunization (non-immune serum used as a negative control). Figure 5 shows that glycated CD59 is present in diabetic kidneys and colocalizes with MAC. The results indicate that 8 out of 13 diabetic subjects (70%) showed glycated CD59 in renal glomeruli whereas none of 7 subjects with other forms of renal disease (sufficient to require a renal biopsy) showed staining.

11. Experiment 6 The anti-glycated CD59 antibody was used to detect the presence of glycated CD59 in diabetic nerves. The samples were paraffin blocks from diabetic patients that underwent a sural nerve biopsy because of diabetic neuropathy. The samples were obtained from a study of diabetic nerves conducted by Dr. Arthur Hays, Chief of Neuropathology at the Columbian Presbyterian Hospital in New York. The paraffin blocks were sectioned, paraffin removed by standard methods and then the thin sections were stained with anti-glycated CD59 antibody, with anti-MAC antibody, with an anti-ULEX antibody that specifically recognizes the human endothelium (this is to label and identify the blood vessels within the nerve) and with the IgG fraction of the rabbit serum extracted before immunization (non-immune serum used as a negative control). Figure 6 shows that the micro-vessels identified by staining with anti-ULEX antibodies also stain positive for glycated CD59 which colocalizes with MAC. In this experiment, 8 out of 12 diabetic subjects (70%) showed glycated CD59 in sural nerves whereas none of the samples from 14 subjects with other forms of neuropathy (sufficient to require a nerve biopsy) showed any staining for glycated CD59.

12. Experiment 7 The anti-glycated CD59 antibody was used to detect the presence of glycated CD59 in diabetic micro-vessels from a diabetic foot. The samples were paraffin blocks from diabetic patients that underwent a tissue biopsy because of diabetic peripheral artery disease. The samples were obtained from a study of diabetic blood vessels conducted by Dr. Michael Conti, at Brigham and Women's Hospital. The paraffin blocks were sectioned, paraffin removed by standard methods and then the thin sections were stained with anti-glycated CD59 antibody, with anti-MAC antibody, with an anti-ULEX antibody that specifically recognizes the human endothelium (to label and identify the blood vessels within the nerve), and with the IgG fraction of the rabbit serum extracted before immunization (non-immune serum used as a negative control). Figure 7 shows that the micro-vessels identified by staining with anti-Ulex antibodies also stain positive for glycated CD59 which colocalizes with MAC. In this experiment, 5 out of 5 diabetic subjects (100%) showed glycated CD59 in micro-vessels whereas none of the samples from 4 subjects with other forms of arterial disease (sufficient to require a biopsy) showed any staining for glycated CD59.

13. Glycated CD59 has been identified in the main target organs of the diabetic complications as well as in urine and plasma of diabetic patients.

14. In view of the presence of glycated CD59 in human diabetic urine and/or plasma, the strong correlation with glycated hemoglobin (HbA1c), the current clinical standard for clinical assessment of glycemic load in diabetics, one of ordinary skill in the art would consider the data presented above as predictive of human diagnostic value and efficacy in assessing glycemic control.

15. Glycated CD59 may mediate the vascular complications of diabetes. In contrast, glycated hemoglobin has no recognized action in the pathogenesis of the disease. In view of the pathogenic role that glycated CD59 and the complement system may play in the development of vascular diabetic complications and the absence of any pathogenic role of HbA1c, one of ordinary skill in the art would consider measurement of glycated CD59 in urine and/or plasma and/or tissue a useful clinical indicator of glycemic control and of the susceptibility of a diabetic subject to develop diabetic vascular complications.

I, José Halperin, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this document and any patent which may issue from the above-identified patent application.

Date: 3-12-03

José Halperin, M.D.

Figure 1

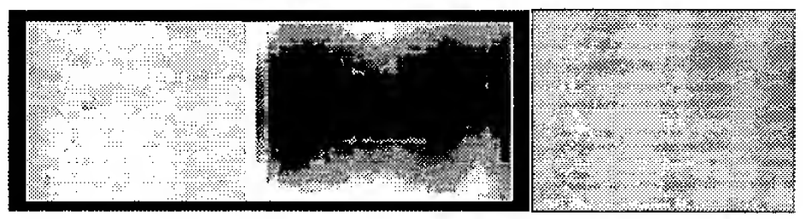
Comparison of human CD59 sequence between residues 36 and 49 in the wild type protein and the synthetic peptide used as antigen for generation of anti-glycated CD59 antibodies

						44			41					
WT human sequence	D	N	F	N	C	H	E	F	K	W	C	K	N	Y

Glycated peptide	D	N	F	N	**A**	H	E	F	**K_g**	W	**A**	K	N	Y

Figure 2

Specificity of the anti-glycated CD59 antibody



Western blot A

OD

CD59	0.083
$CD59_{glu}$	0.212
Alb_{glu}	0.078

ELISA B

Figure 3

Glycated CD59 in Diabetic Urine

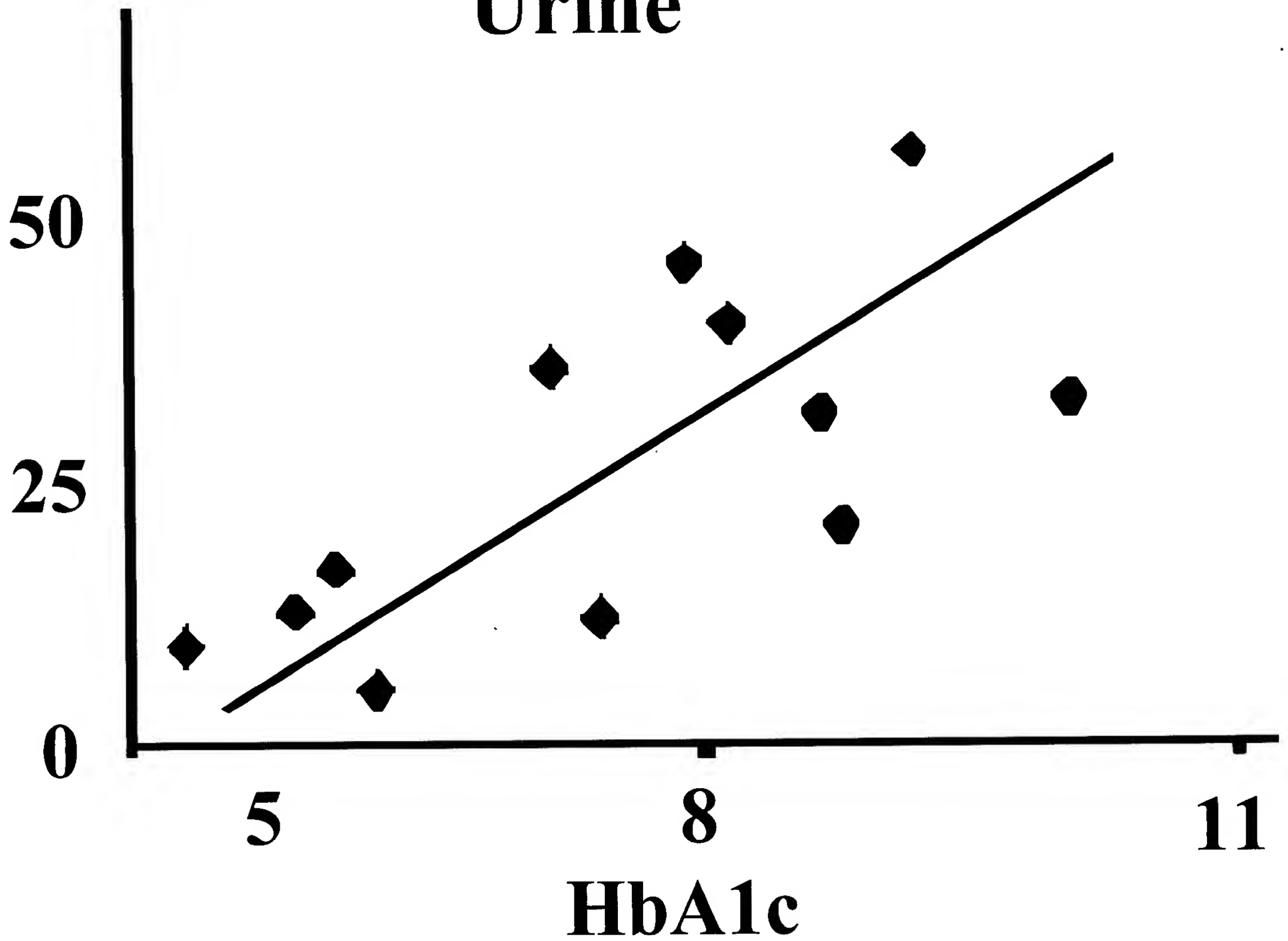


Figure 4

Glycated CD59 in Diabetic Plasma

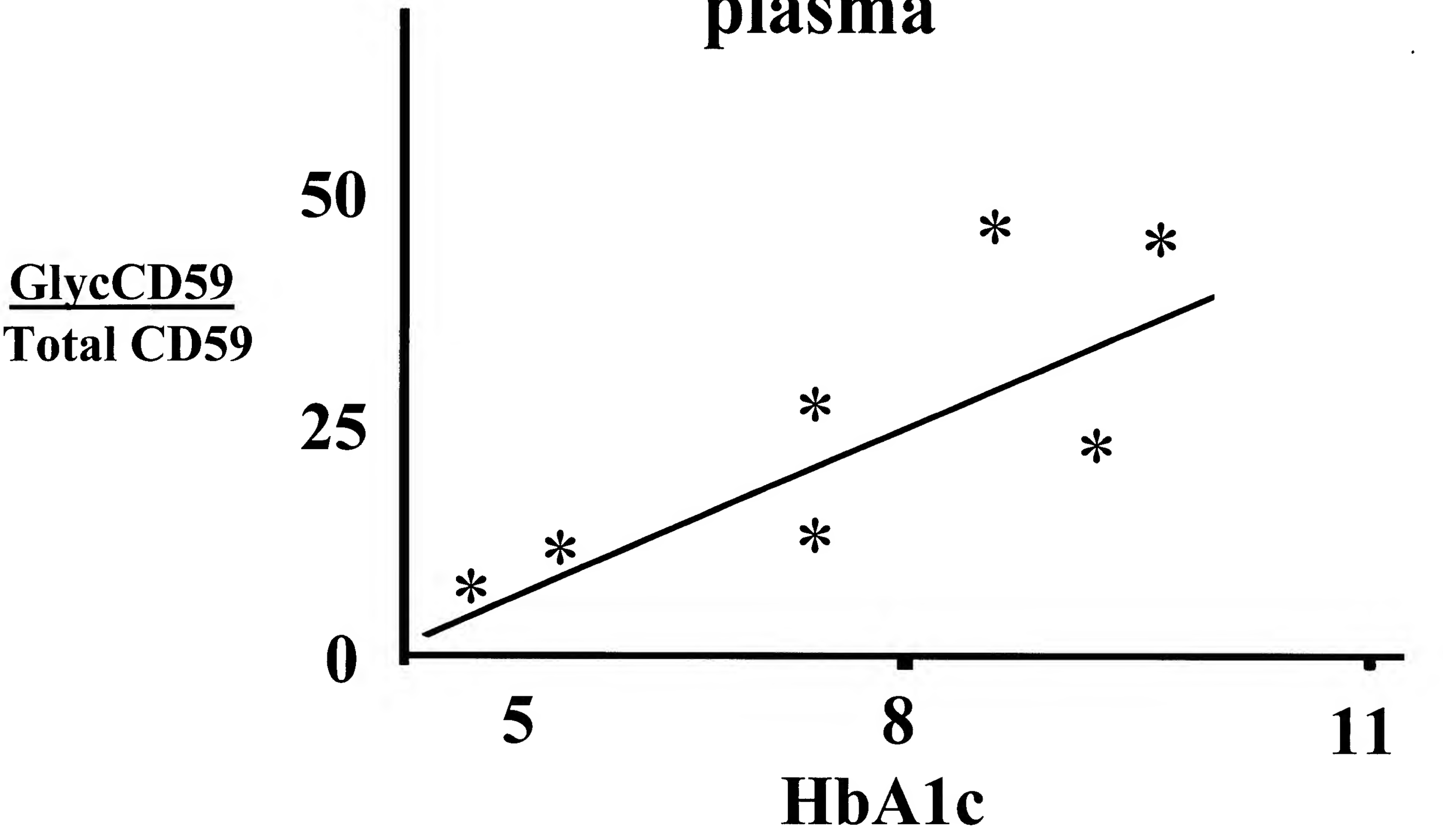
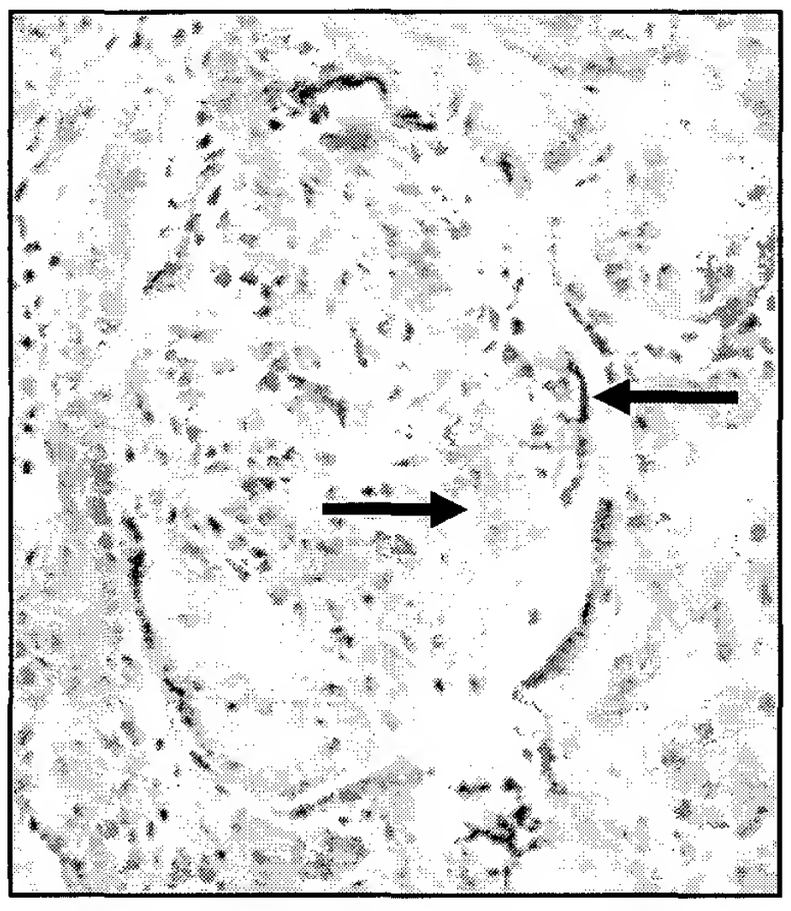


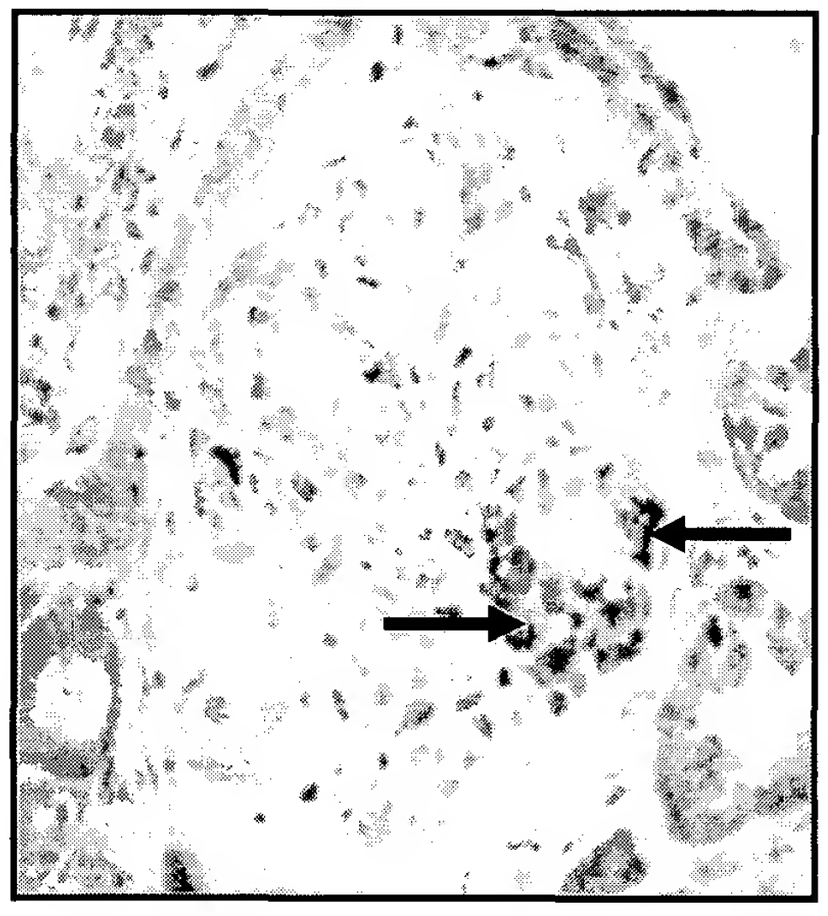
Figure 5

Diabetic nephropathy

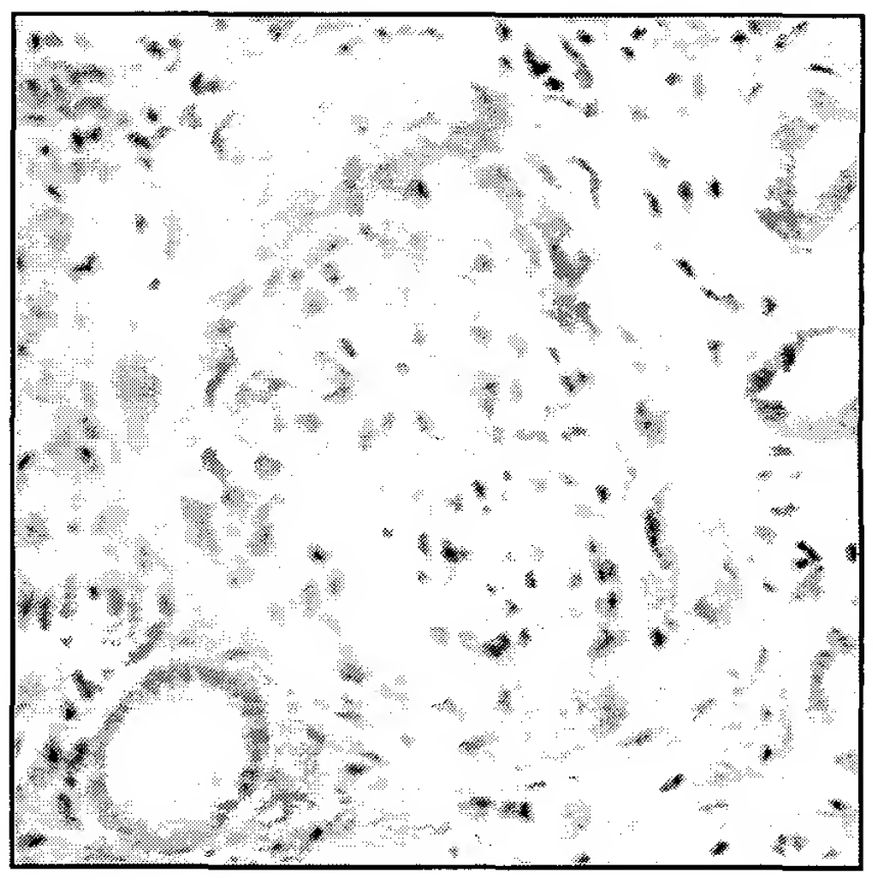
serial sections



Anti-MAC



Anti-CD59glu



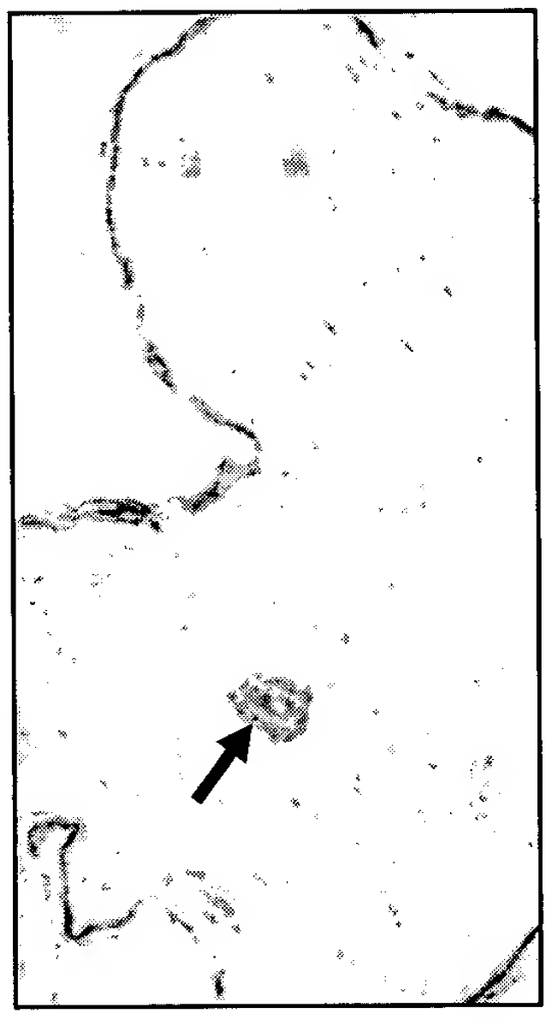
Non-immune IgG

Figure 6

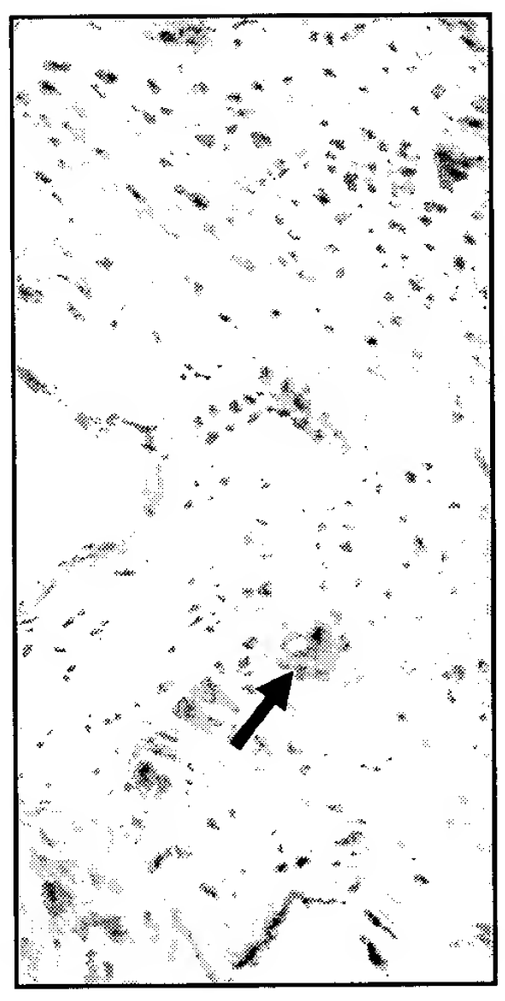
Diabetic neuropathy

serial sections

ULEX



Anti-MAC



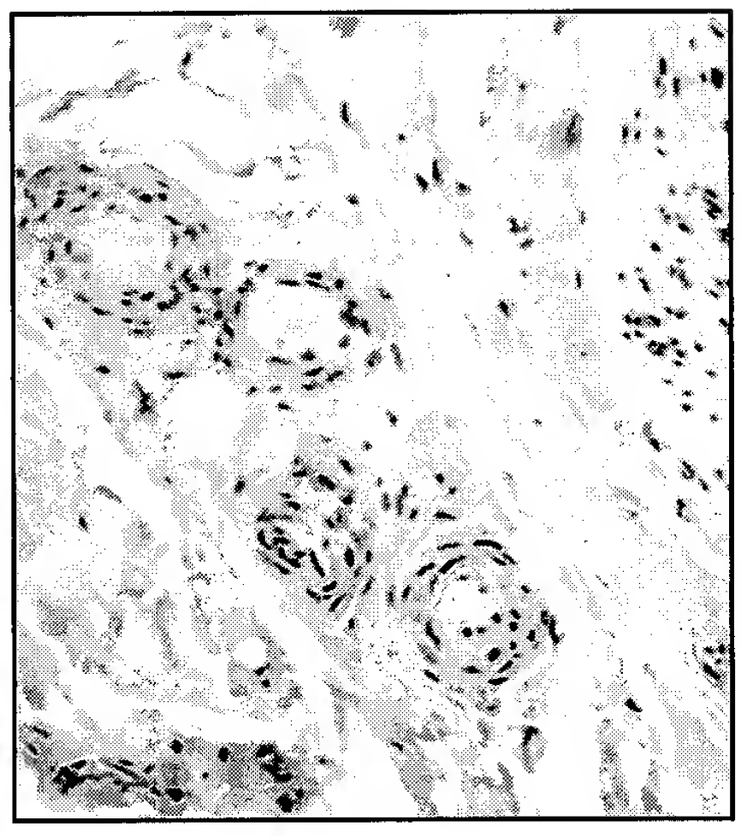
Anti- CD59glu

Non-immune IgG

Figure 7

Diabetic microvascular disease

serial sections



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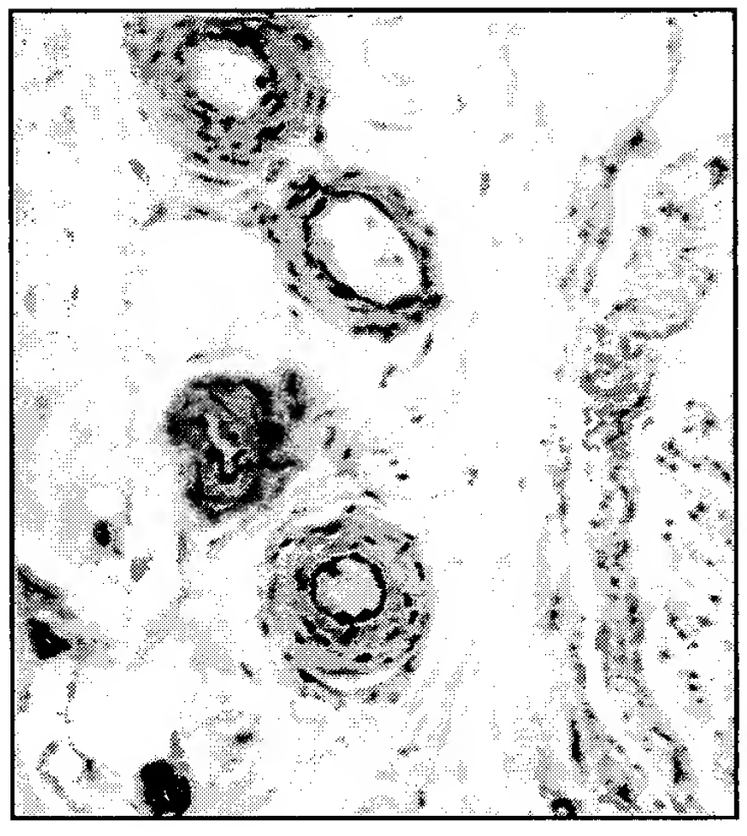
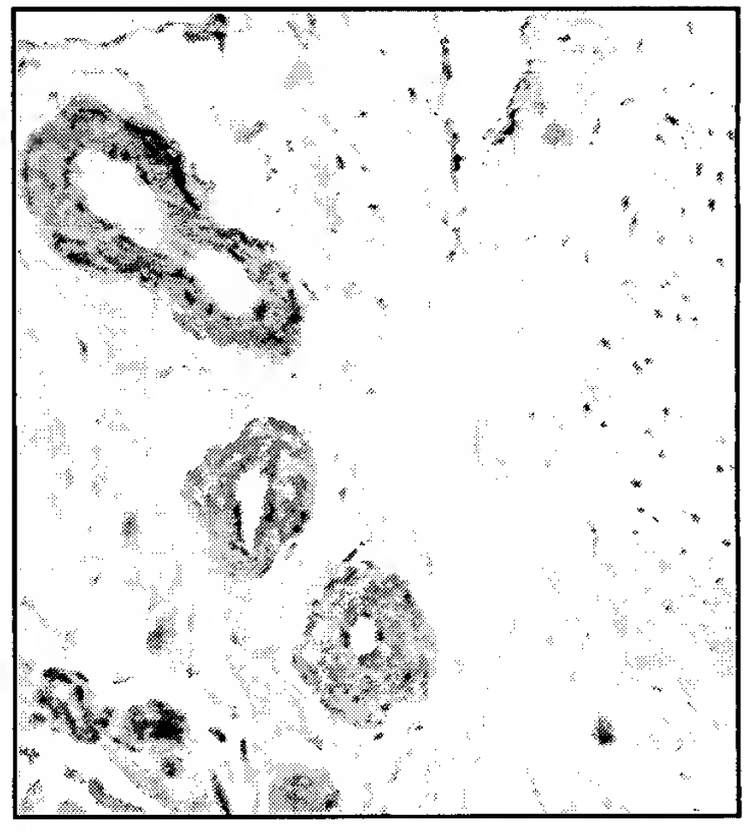


Anti-MAC



Anti-CD59glu